

CLAIMS

- 5 1. A method of amplifying, and optionally also detecting, a target nucleic acid sequence, the method comprising the steps of:
- 10 a) providing a sample that may or may not comprise a target nucleic acid sequence,
- 15 b) providing a pair of outer primers and a pair of inner primer, a nucleic acid polymerase and standard reagents for PCR, the melting temperature (T_m) of the pair of outer primers being at least 2 °C higher than the T_m of the pair of inner primers,
- 20 c) contacting the sample with the pair of outer primer and the pair of inner primers, and standard reagents for PCR, thus obtaining the reaction mixture,
- 25 d) cycling, at least two times, the temperature of the reaction mixture between a first denaturation temperature, a first annealing temperature and a first extension temperature, the first annealing temperature being similar to or lower than the lowest T_m of the outer primer pair and higher than the highest T_m of the inner primer pair,
- 30 e) cycling, at least two times, the temperature of the reaction mixture between a second denaturation temperature, a second annealing temperature and a second extension temperature, the second annealing temperature being similar to or lower than the lowest T_m of the inner primer pair,
- 35 f) optionally, analysing the product of step d and/or step e) to detect the presence of the target nucleic acid sequence.
2. The method according to claim 1, wherein at least one primer of the outer primer pair comprises a T_m -increasing component.
3. The method according to claim 1 or 2, wherein both of the primers of the outer primer pair comprise a T_m -increasing component.
4. The method according to claim 3, wherein the T_m -increasing component binds non-
40 specifically to nucleic acids.

5. The method according to any of claims 2-4, wherein the T_m-increasing component comprises one or more moieties selected from the group consisting of a modified nucleotide and a minor groove binding agent.
- 5 6. The method according to claim 5, wherein the modified nucleotide is a peptide nucleic acid (PNA) or a locked nucleic acid (LNA).
7. The method according to any of claims 2-6, wherein the T_m-increasing component increases the T_m of the primer with at least 1°C relative to the T_m of the same primer not
10 comprising the T_m-increasing component.
8. The method according to any of the preceding claims, wherein the second denaturation temperature is at least 1°C lower than the first denaturation temperature.
- 15 9. A method for detection of *Bacillus anthracis*, the method comprising detecting a target nucleic acid sequence according to the method of claim 1-8, the target nucleic acid sequence being specific for the pXO1 or pXO2 plasmid of *Bacillus anthracis*, wherein the pair of outer primers and the pair of inner primers are selected from the pXO1 or pXO2 plasmid of *Bacillus anthracis*.
- 20 10. The method according to claim 9, wherein the pair of outer primers and the pair of inner primers are selected so as to amplify a target nucleic acid sequence related to a gene selected from the group of *B. anthracis* genes consisting of capA gene, the capB gene, the capC gene, the lef gene.
- 25 11. The method according to claim 9 or 10, wherein target nucleic acid sequence is related to the capA gene and
- a primer of the pair of outer primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 1, SEQ ID NO: 2, a homologous sequence thereof, and a complementary sequence thereof, and
- 30 - a primer of the pair of inner primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 3, SEQ ID NO: 4, a homologous sequence thereof, and a complementary sequence thereof.
- 35 12. The method according to any of the claims 9-11, wherein target nucleic acid sequence is related to the capA gene and the pair of outer primers comprises SEQ ID NOs: 1 and 2 and/or the pair of inner primers comprises SEQ ID NOs: 3 and 4.
- 40 13. A kit comprising a pair of outer primers and a pair of inner primer, the melting temperature (T_m) of the pair of outer primers being higher than the T_m of the pair of inner primers.

14. The kit according to claim 13, wherein at least one primer of the outer primer pair comprises a T_m-increasing component.
15. The kit according to claim 13, wherein both of the primers of the outer primer pair
5 comprises a T_m-increasing component.
16. The kit according to any of the claims 13-15, wherein the T_m-increasing component binds non-specifically to nucleic acids.
- 10 17. The kit according to any of the claims 13-15, wherein the T_m-increasing component comprises one or more moieties selected from the group consisting of a modified nucleotide and a minor groove binding protein.
- 15 18. The kit according to claim 17, wherein the modified nucleotide is a peptide nucleic acid (PNA) or a locked nucleic acid (LNA).
19. The kit according to any of the claims 13-18, wherein the T_m-increasing component increases the T_m of the primer with at least 1°C relative to the T_m of the same primer not comprising the T_m-increasing component.
- 20 20. Kit for detection of *Bacillus anthracis*, the kit comprising a pair of outer primers and a pair of inner primer, the melting temperature (T_m) of the pair of outer primers being higher than the T_m of the pair of inner primers, wherein the pair of outer primers and the pair of inner primers are selected from the pXO1 or pXO2 plasmid of *Bacillus anthracis*.
- 25 21. The kit of claim 20, wherein the pair of outer primers and the pair of inner primers are selected so as to amplify a target nucleic acid sequence within a gene selected from the group of *B. anthracis* genes consisting of capA gene, the Cap B gene, the Cap C gene, the lef gene.
- 30 22. The kit according to claim 20 or 21, wherein target nucleic acid sequence is related to the capA gene and
- a primer of the pair of outer primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 1, SEQ ID NO: 2, a homologous sequence thereof, and a complementary sequence thereof, and
- 35 - a primer of the pair of inner primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 3, SEQ ID NO: 4, a homologous sequence thereof, and a complementary sequence thereof.
- 40 23. The kit according to any of the claims 20-22, wherein target nucleic acid sequence is related to the capA gene and the pair of outer primers comprises SEQ ID NOs: 1 and 2 and/or the pair of inner primers comprises SEQ ID NOs: 3 and 4.

24. An analysis system for detection of a microorganism, the analysis system comprising a pair of outer primers and a pair of inner primer, the melting temperature (T_m) of the pair of outer primers being higher than the T_m of the pair of inner primers.
- 5 25. The analysis system of claim 24, wherein the analysis system is selected from the group consisting of a lateral flow device, a biochip, and a microarray.